Endothelial Nitric Oxide Function and Tubular Injury in Premature Infants

Huseynova Saadat, PhD
Department of Neonatology
Azerbaijan Medical University
Bakikhanov 23, Baku, Azerbaijan

Akhundova Afag
Department of Neonatology
Azerbaijan Medical University
Bakikhanov 23, Baku, Azerbaijan

Panakhova Nushaba, PhD
Department of Neonatology
Azerbaijan Medical University
Bakikhanov 23, Baku, Azerbaijan

Hasanov Safikhan, MD
K. Farajova Pediatry Institute
Department of Neonatology
Azerbaijan Medical University
Bakikhanov 23. Baku, Azerbaijan, AZ 1022

Ali-zade Samaya, PhD
Pediatry Faculty of Odlar Yurdu University
Baku, Azerbaijan, AZ 1065

Abstract

The aim of this work was to determine the association between renal endothelial activation and tubular epithelial injury in low-birth-weight (LBW) and in very-low-birth-weight (VLBW) infants with hypoxic-ischemic encephalopathy (HIE). Urine samples from all of the infants were assessed for kidney injury molecule-1 (KIM-1) antigen and nitric oxide (NO) at their 1st and 7th days of life. The mean urine KIM-1 concentration was higher (1.5±0.4 ng/ml) in LBW infants compared with VLBW infants (0.8±0.4 ng/ml) and tended to decline in both groups of infants. In LBW infants, urine NO levels did not change, and the decline of NO was noted in the VLBW infants, decreasing from 418.4±84.54 µM/L to 261.2±20.2 µM/L. We observed the decease of KIM-1 concentration in a background of decreased nitric oxide metabolites , which may indicate low endothelium activation in response to ischemia-reperfusion and confirm the low probability of epithelial injury in VLBW infants.

Keywords: Endothelial dysfunction, renal injury, preterm infants, intrauterine hypoxia, tubular necrosis

Introduction

The fetal response to an episode of asphyxia is to preserve perfusion and oxygenation of the heart, brain and adrenal glands at the expense of the other "non-vital" organs such as the kidney, lungs, gastrointestinal tract and musculoskeletal system (Craig et al., 2000). The incidence of single or multiple organ injury in association with neonatal encephalopathy varies from 40 to 100% and appears to be correlated with the severity of neuronal injury (Philip, 2009). Multiple organ injury appears in the context of endothelial cell dysfunction, which is a common precursor and denominator of various pathological conditions in newborn infants (Fabian et al., 2008).

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Endothelial cells become activated during reperfusion following the initial cerebral ischemic event, which results in the production of biologically active molecules such as endothelins, nitric oxide (NO) and several types of chemotactic molecules (Goligorsky, 2000). NO is a vasodilator produced by endothelial nitric oxide synthase and plays an important role in the regulation of vascular tone and blood flow in various organs . The renal system is often involved, and clinical findings of kidney injury range from mild oliguria, proteinuria and hematuria to renal tubular necrosis and acute renal failure (Baylis, 2008).

There are several sensitive and specific biomarkers for the early detection of kidney injury (Du et al., 2011). The urinary kidney injury molecule-1 (KIM-1) level serves as a noninvasive, rapid, sensitive and potentially high-throughput method for the early detection of kidney injury in pathophysiological studies . The KIM-1 protein is expressed at very low levels in the normal rodent kidney, but expression increases dramatically after injury in proximal tubule epithelial cells in ischemic acute renal failure .

There are many ways in which NO deficiency can affect renal function, and it is likely that multiple mechanisms are involved in the development of glomerular and tubular injury. The aim of this study was to determine the association between renal endothelial reaction and tubular epithelium injury in preterm infants with hypoxic-ischemic encephalopathy (HIE). We examined the hypothesis that impaired endothelial function is associated with more severe tubular injury and high levels of KIM-1 expression in preterm infants with perinatal HIE.

Subjects and methods

Patients. This work is a component of a nonrandomized prospective study designed to identify the roles of perinatal factors in the neurological and somatic health of preterm infants. The study protocol was reviewed and approved by the Problem Commission on Pediatric Research at Azerbaijan Medical University in June 2011, and by the National Committee on Bioethics, Ethics of Science and Technology. Newborns with gestational ages of 27-36 weeks and birth weights of 970-2,500 g, delivered at the Sh. Aleskerova Maternity Hospital (the educational unit of Azerbaijan Medical University) between January and November 2011, were recruited to participate in this study. We included 47 infants with hypoxic-ischemic encephalopathy in the study. The exclusion criteria were clinical or laboratory evidence of congenital infection, neonatal sepsis, or congenital malformation. Gestational age was assessed by the last menstrual period and ultrasonography and confirmed using the scale of *Ballard et al*. The newborns were allocated into two groups: 29 infants weighing more than 1,500 g were assigned to the low-birth-weight (LBW) group, and 18 infants weighing less than 1,500 g were assigned to the very-low-birth-weight (VLBW) group. Acute kidney injury (AKI) was diagnosed using a categorical definition based on serum creatinine level . The diagnosis of HIE was based on the *Levene* classification (Levene, 1995). Brain injury was also confirmed by cranial ultrasound, which was performed in all infants at the 2nd and 6th days of life with 5- and 7.5-MHz vector transducers. The neurological assessment was performed according to the *Dubowitz/Ballard* scale during the neonatal period.

Urine collection. Urine samples were collected from all newborns on the 1st and 7th days of life. For routine urine KIM-1 antigen and NO assessment, urine samples were frozen at -20°C at the time of collection.

Nitric oxide assay. Urine nitric oxide concentrations were quantified with a commercial kit (Thermo Scientific, USA). The principle of the test is based on the conversion of nitrate to nitrite by the nitrate reductase enzyme. Nitrite is then detected using a method based on the Griess reaction by monitoring the absorbance of visible light at 540 nm. Urine samples were diluted with 1X diluent reagent, ultrafiltered through a 10,000 MWCO filter and used directly in the assay. Nitrite concentrations were determined using a nitrite standard curve. The sample nitrate was calculated by subtracting the sample nitrite concentration from the measured nitrite concentration after the enzymatic conversion of nitrate.

KIM-1 assay. Urine KIM-1 levels were detected in the urine samples using an ELISA test kit (Argutus Medical, BioAssay Works, Ireland). Urine samples were diluted 1:3, and results were multiplied by the dilution factor. KIM-1 results were read using an ELISA plate reader set at 405 nm with a 490-nm differential filter.

Statistical analysis. Analyses were performed using SPSS version 17 and Stata version 11. In all instances, significance was established at $p < 0.05$. Student's *t*-test and the Mann-Whitney test were used to compare parametric and non-parametric parameters. Results are expressed as means ± SEM, or as ranges where appropriate. Categorical variables were analyzed using χ^2 or Fisher's exact test as appropriate.

Results

Table 1 lists the characteristics of the study participants. There were no significant differences in gender or maternal age between the groups, but significant differences were observed for (low) Apgar score at the 1st minute, pre-eclampsia, multiple pregnancy and neonatal mortality. High-frequency intrauterine growth restriction (IUGR) was diagnosed in the VLBW infants. Acute kidney injury developed in 3 (10.3%) of the LBW and in 2 (11.76%) of the VLBW infants.

Table 2 presents the KIM-1 and nitrite/nitrate concentrations in the urine of the infants at the beginning and end of the early neonatal period. The urine KIM-1 concentration was higher (1.5±0.4 ng/mL) in the LBW infants compared with the VLBW infants (0.8±0.4 ng/mL) and tended to decline over time in both groups. We calculated nitric oxide concentrations according to the nitrate/nitrite data, and urinary nitric oxide levels did not differ in relation to body weight on the first day of life. NO concentrations were measured at 408±41.14 µM/L in the LBW and 418.4±84.54 µM/L in the VLBW infants. The nitrate concentration remained stable at a high level in the LBW group, and at the end of the early neonatal period, the mean NO concentration was 386.2±61.5 µM/L. In the VLBW infants, nitrate diminished significantly, decreasing from 418±85.54 µM/L to 261.2±20.2 µM/L, compared with the LBW infants, and statistically significant differences were noted not only between the 1st- and 7th-day parameters ($p<0.01$) but also between the LBW and VLBW infants ($p<0.05$).

Discussion

AKI in preterm infants with HIE occurs with a frequency depending on gestational age, and several specific biomarkers are known as precursors of renal failure in premature infants (Askenazi et al., 2011). As suggested in several previous studies, urinary nitrate may be an early biomarker of renal dysfunction in pediatric and adult populations (Mian, 2011). KIM-1 is an indicator of renal tubular epithelium damage of various origins (Du et al., 2011). Our study did not specifically include children who developed acute renal failure. The main purpose of our investigation was to study the relation between renal tubular epithelium injury and vascular endothelial activity in preterm infants exposed to hypoxia-ischemia. Plasma and urine levels of nitric oxide vary depending on the severity of hypoxic injury and gestational age. The urine nitrate level can be used as a unique indicator of endothelial damage to kidney vessels after ischemia-reperfusion. We determined the variable nitrate concentrations in the urine of preterm infants and found that nitrate was significantly reduced during the early adaptation of VLBW infants compared with the LBW infants. NO reduction during the early adaptation period of hypoxia-exposed VLBW children may indicate a low inflammatory response in the organ in general and in the renal vascular endothelium in particular. Low inflammatory response may be related to the incomplete formation of inflammatory components in the context of morphological and functional immaturity, or it could be the result of a low endogenous NO synthase activity. Our findings are supported by those of *Elli et al*., who reported diminished NO production associated with the prevention of AKI.

Recent studies have described low baseline values of urine KIM-1 with increasing gestational age in infants with AKI. Higher levels of KIM-1 were found in infants with extremely low birth weights, and statistically significant values were found for the KIM-1/creatinine ratios of preterm infants (Askenazi et al.). We found contradictory results in the VLBW infants with hypoxic encephalopathy and without AKI. Our results show a decline of KIM-1 concentration in a background of low nitric oxide activity as an early neonatal adaptation in VLBW newborns affected by hypoxia-ischemia but without AKI. Low KIM-1 levels may indicate the low probability of epithelial injury on the basis of low endothelial activation in VLBW infants. Such weakened endothelium activation may help prevent tubular necrosis and AKI in VLBW infants.

This study has several limitations. For the correct assessment of endothelial function in relation to gestational age, it would be desirable to investigate infants in both research groups in separate subgroups on the basis of IUGR syndrome. However, the number of IUGR infants in each group was insufficient to obtain statistically reliable results. In addition to KIM-1 levels, the investigation of other sensitive markers of renal injury in association with endothelial dysfunction should lead to a more comprehensive interpretation of renal function and extrauterine adaptive mechanisms of the kidneys. Recently, *La M. Gaetano* and coauthors reported that the level of neutrophil gelatinase-associated lipocalin at birth is a more sensitive predictor of early renal function than KIM-1 in VLBW infants.

In conclusion, adaptive mechanisms of the kidneys to hypoxia and ischemia-reperfusion in infants depend on their gestational age and maturity. Endothelial activation is not perfect in VLBW infants, resulting in low cerebral blood flow and worse neurological outcomes. However, in this case, a low endothelial response to hypoxia is associated with reduced tubular injury, which provides a basis for the estimation of this change as a positive adaptive mechanism for the maintenance of renal function and the prevention of AKI in infants with incomplete nephrogenesis.

Table 1. Neonatal and maternal characteristics of the study groups.

	LBW group (N=29)	VLBW group (N=17)
Infant characteristics		
Birth weight (g)	2330.2±91	1261.1±69.6*
GA (wk)	35.9±0.8	32.9±0.7*
Apgar score at 1 min	5.33±0.33	4.4±0.51*
Apgar score at 5 min	6.33±0.31	5.50±0.5
Male	11 (37.93)	7 (41.17)
Female	18 (62.07)	10 (58.82)
IUGR	4 (13.79)	7 (41.18)*
Acute kidney injury	3 (10.34)	2 (11.76)
Neonatal mortality	1 (3.45)	4 (23.53)*
Maternal characteristics		
Age	24±1.8	27±2.4
Maternal pre-eclampsia	4 (13.79)	7 (41.18)*
Multiple pregnancy	3 (10.34)	5 (29.41)*
Cesarean section	6 (20.69)	6 (35.29)

Data are expressed as the mean ± SEM and as n (%); *p<0.05

Table 2. Changes in urine KIM-1 and urine NO levels in LBW and VLBW infants.

	LBW infants (N=29)		VLBW infants (N=17)	
	1st day	7th day	1st day	7th day
KIM-1, ng/mL	1.5±0.4 (0.3-9.9)	1.1±0.3 (0.3-4.8)	0.8±0.4 (0.3-3.3)	0.4±0.1 (0.3-0.6)
Urine NO, µM/L	408±41.14 (64-692.4)	386.2±61.5 (72-629.2)	418.4±84.54 (155.2-611.2)	261.2±20.2*¶ (85-420.8)

Data are expressed as the mean ± SEM and interquartile ranges; *p<0.05 compared with first-day parameters, ¶p<0.05 compared with LBW infants.

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